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A NOVEL GREEN ASSESSMENT ANALYTICAL METHOD TO QUANTIFY THE CONTENT OF DIMETHYLGLYOXIME IN LENALIDOMIDE USING LC-MS

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ABSTRACT

A robust LC-MS method is established for the separation and quantification of Dimethylglyoxime foreign substance in Lenalidomide. Chromatographic separation was accomplished using a Zorbax Eclipse C18, 100 X 4.6 mm, 3.5 µ column. A Mixture of 0.01M of Ammonium formate as 50v/v and Acetonitrile as 50 v/v used as the mobile phase. The rate of flow is measured at 0.40mL/min with Gradient elution and mass spectrometry operating in positive ion mode. Selectivity of the method proved that protonated species of Dimethylglyoxime (m/z 117.16) separated from Lenalidomid drug substances, with a exceptional linearity over a range of 6-120 ppm, limits of detection of 2.00 ppm and quantification of 6.05 ppm. Consistent recovery rates were obtained between 90-100%, and the analytical solution remained stable for up to 72 hours at 2-8ºC. Subsequently, the developed and validated analytical method was assessed for environmental sustainability using established greenness assessment tools. The AGREE score was calculated at 0.62, and the BAGI value at 70.0. These values reflected a favorable environmental profile, efficient sample preparation, and practical sustainability. These proposed separation strategies showed high specificity, substantiating clear resolution of DMG foreign substance by the drug compound, also with foreign substances. Together, these metrics confirmed the method's suitability for both regulatory compliance and routine application in industrial Quality Control laboratories.

Keywords: Lenalidomide, Dimethylglyoxime, Selective Ion Monitoring, AGREE, BAGI Greenness.
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INTRODUCTION

Lenalidomide (LID) (referred to as CC-5013) is an immunomodulatory drug substance with potent antineoplastic, anti-angiogenic, and anti-inflammatory properties.[1] LID endures as a racemic mixture of the active S(-) and R(+) forms.[2] Anyhow, LID is safer and headier than that of thalidomide, having fewer adverse effects and toxicities.[3] Thalidomide and its analogues, including LID, are referred to as immunomodulatory imide drugs, which are well known as cereblon modulators. LID works through different mechanisms of action that promote malignant cell death and enhance host immunity.[2] This is available in the market as oral capsules. LID is approved by the FDA for the treatment of lymphoma in selected patients.[4] Because of severe teratogenicity, pregnancy should be avoided prior to the start of treatment, and patients should enroll in the LID REMS program to secure contraception adherence.[5] DMG is a white, crystalline solid by molecular formula $C_4H_8N_2O_2$, and it is commonly used as a reagent in analytical chemistry, particularly for the detection of nickel and palladium. It is also used in ligand competition techniques to determine the concentration of free metal ions in natural waters.[6] The Dimethylglyoxime (DMG) and LID drug substance structure is shown in Fig.-1. As per the route of synthesis of LID described in Fig.-2.[8] Palladium (Pd) was used for NO_2 to NH_2 conversion. Along with 10% w/w charcoal, is 30 min successfully reduced the palladium content in the LID sample. However, DMG has a potential foreign substance synthesis alerting structure. Hence, the foreign substance is controlled as ICH M7 at a specification considered 60 ppm based on the LID maximum daily dose of 25 mg.[9] This regulatory requirement highlights the significance of quantification of DMG content in LID drug substance. ICH and other regulatory bodies have confirmed that the DMG foreign substance should

be below certain thresholds.[9,10] The structure of DMG is less UV active and thermally unstable, and the molecular weight is also low, 116.12 g/mol.[7] Any reported literature was not available. Based on that, the LC-MS technique is selected in Q1 (Multiple ion mode). There are no other standard methods were accessible for the quantification of DMG content present in the LID.[11,12,13]

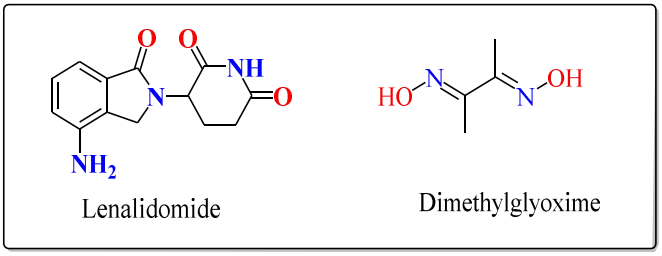

Fig.-1: Chemical Structures of Lenalidomide and DMG

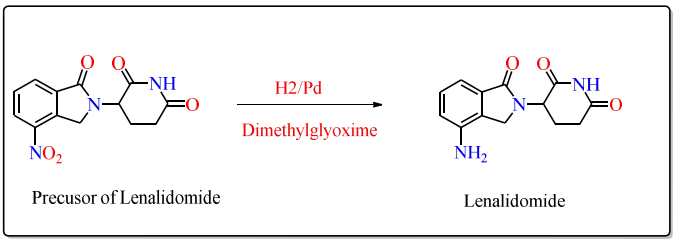

Fig.-2: Route of Synthesis of LID From Precursor

The method development and validation were performed following ICH Q2 (R2) guidelines.[14] Tools such as AGREE and BAGI have emerged to quantify the sustainability of analytical methodologies.[15,16] Incorporating such metrics ensures not only compliance with analytical standards but also aligns with global sustainability goals outlined by ICH Q14[17] and ISO 14001[18] frameworks. This work demonstrated the critical role of LC-MS in advancing foreign substance profiling, emphasizing its contribution to pharmaceutical QC and regulatory compliance while addressing the challenges of genotoxic foreign substance detection.

EXPERIMENTAL

Chemicals and Reagents

LID drug substance, DMG foreign substance, and other foreign substances were obtained from Reputed Pharma Company located in Visakhapatnam. All the chemicals, reagents and solvents utilized in this work are analytical grade and garnered by Merck India. Ultrapure water was garnered from the Milli-Q system from Millipore.

Instruments

The LC-MS/MS used was an AB Sciex 3200 Q-Trap with Electrospray MS operating in positive ion mode in Q1(Multiple ion) and monitored the protonated species of DMG (m/z 117.16) and an LC Agilent 1200 by quaternary pump, degasser, column oven, auto-sampler, as well as a PDA detector. Backlashes by detectors are surveilled and penalized by utilizing analyst software. Measurements of weight are sophisticated by utilising a RADWAG analytical balance (Model AS 82/220.R2 PLUS).

Preparation of Solutions

A mixture of 0.01M of Ammonium formate as 50v/v and Acetonitrile as 50 v/v utilized as the mobile phase as well as the diluent. Standard stock solution is prepared by weighing and trasferring10.0000mg of DMG standard into a 100.00mL standard flask contains 50.00mL diluent, mixed well and then marked to the mark with a suitable diluent. Further, 3.00ml above the standard stock solution was transferred to a 100.00 mL standard flask, and then made up to the mark with the suitable diluent, again 1.00mL of this diluted solution was transferred to a 10.00 mL standard flask and made up to the mark with the suitable diluent. Test Sample Solution prepared by transferring 250.00mg of LID into a 50.00mL standard flask. mixed and made up to the mark with diluent.

Method Validation

Zorbax Eclipse C18; 100 X 4.6 mm; 3.5µm stationary phase is utilized to separate DMG foreign substance by LIDe substance. A Mixture of 0.01M of Ammonium formate as 50v/v and Acetonitrile as 50 v/v used as the mobile phase, pumped by a gradient program with optimized chromatographic conditions. 0.40mL/min is the rate of flow. 25ºC is the temperature. The column oven was 40ºC. Volume of the sample injected is 10µL in a time interval of 27 minutes. Run time. Mass conditions Scan Q1 Multiple Ions, Dwell time 200 milli sec., Curtain gas 30 psi, Ion spray voltage 5500 V, Temp. 500 °C, Ion Source GS1 40 psi, Ion Source GS2 45 psi, Ion Source Heater on, Decluttering potential 31 V, 8V is Entrance potential, 16 V is Collision cell entrance potential, Valco valve Condition 9.3 at position B and 12 at position A.

RESULTS AND DISCUSSION

System Suitability/System Precision

This parameter is performed by using a 60 ppm standard solution. The obtained values are represented in Table-1. A total of six replicate injection responses are taken. The % RSD is measured as 1.52%. This indicates excellent repeatability as well as consistency in the response of the peak for DMG. These results substantiate that this system met acceptance criteria, as the % RSD was below to 10.0%. This cinches that this LC-MS process is highly suitable for beyond the analysis of various samples within the specified strength range.

Table-1: Precision of 60 ppm DMG Standard Solution

Injection No	Retention time (min)	Response
1	8.41	155000
2	8.41	155000
3	8.38	155000
4	8.40	153000
5	8.40	149000
6	8.41	153000
Average	8.40	153333
STDEV	0.01	2338.09
% RSD	0.12	1.52

Specificity

Specificity is conclusive by preparing after injecting total foreign substances, including individual diastereomers, as well as specified foreign substances (USP monograph foreign substances), such as DMG. It confirmed that the DMG method was selective for the determination of DMG with respect to other specified foreign substances. Results are summarized in Table-2. The DMG was not detected in the LID sample solutions, but was found in the foreign substance-spiked sample. It indicates no interference from other foreign substances at the specification level. Similarly, these results demonstrate that the analytical method can accurately distinguish and quantify the DMG foreign substance in LID in the presence of potential interfering substances, ensuring reliable analysis of samples in pharmaceutical formulations.

Table-2: Specificity Results

Name	Rt of foreign substance in min.	Rt of foreign substances in Spiked solution in min.
DMG	8.40	8.40
LID	10.30	10.30
LID Foreign substance-A	Not detected	Not detected
LID Foreign substance-B		
LID Foreign substance-C		
LID Foreign substance-D		
LID Foreign substance-E		
LID Foreign substance-F		

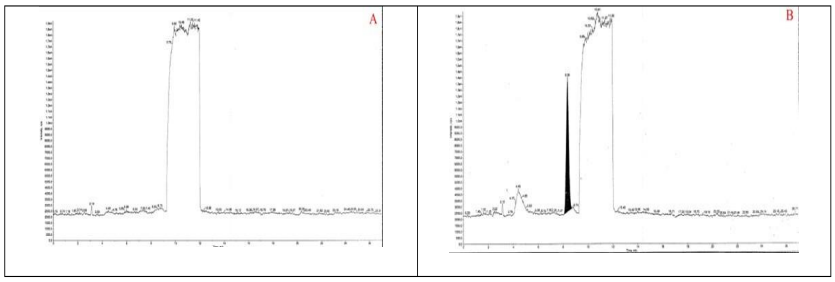

Fig.-3: (A) Blank Chromatogram, (B) Standard Solution Chromatogram

Linearity

For this parameter, a total of eight strength levels of DMG foreign substance standard solutions, which are ranges 6.05ppm to 120ppm. The outcomes clearly demonstrated a strong linear relationship among standard response as well as strength, as indicated by R^2 consistently exceeding 0.999 for total strength levels. The values of peak response at each and every strength level align very closely with values expected, affirming this method's capability to quantitatively measure DMG within a specified range by LOQ up to 200% of the specification limit. Results, displayed in Table-3 and Fig.-4, visually represent these findings.

Table-3: Linearity Response

Concentration (ppm)	Response
6.05	17783
15	39850
30	82600
45	121000
60	158500
75	205500
90	242500
120	325833
correlation coefficient	0.999
slope	2706
% intercept	0.01

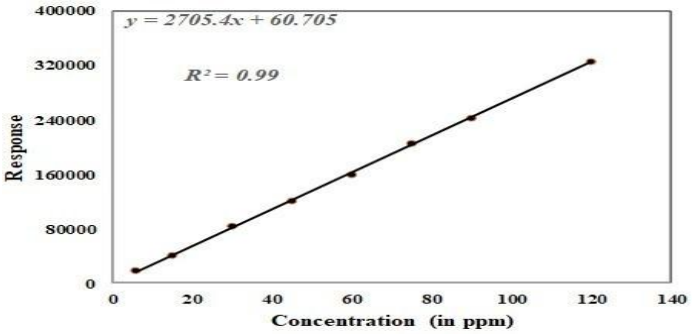

Fig.-4: Linearity Curve for the DMG Foreign Substance from 6.05 to 120 ppm

Detection Limits and Precision at Limit of Quantification (LOQ)

These parameters also identified that s/n value 4.37 at a strength 2.0 ppm consists of a response of 6630, and s/n value 10.3 at a strength 6.0 ppm, containing a response of 17600. The precision of this process is assessed by measuring the peak response of the DMG foreign substance at LOQ. Results so obtained are represented in Table-4 summarizes total replicate injections at the LOQ level. Calculated the average peak response. The values so obtained showed good precision with a %RSD of 6.23%. These values of this method's capability to quantify DMG foreign substance at low levels precisely. Both the chromatograms are displayed in Fig.-5.

Table-4: Precision at LOQ Levels

Repetition No	Response
	LOQ (6.05 ppm)
1	17600
2	16400
3	19200
4	17000
5	19000
6	17500
Average	17783
STDV	1107.09
%RSD	6.23

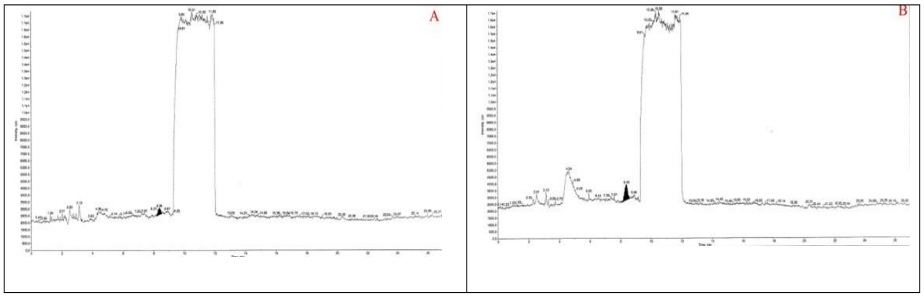

Fig.-5: (A) DMG Foreign Substance at LOD level, (B) DMG Foreign Substance at LOQ Level

Precision (Method Precision and Intermediate Precision)

This parameter is performed by repeatability solutions of spiked samples at a specification level of 60 ppm. A total of six spiked samples are subjected to analysis for determining the method precision, as well as %RSD values identified as 2.14%. The %RSD values are 2.12%. Altogether, the %RSD for the twelve spiked sample found as 2.04%. These findings accentuate this method's reliability as well as suitability to quantify DMG foreign substance at the specified strength level accurately, meets required precision criteria. Above all, outcomes were represented in Table-5.

Table-5: Method Precision and Intermediate Precision Results

Preparation	DMG foreign substance content (in ppm)	
	Intermediate precision	Method precision
1	59	58
2	58	58
3	58	59
4	56	56
5	57	58
6	56	56
Average	57	57
Stdev.	1.21	1.22
% RSD	2.12	2.14
Average	57	
Stdev.	1.16	
% RSD	2.04	

Accuracy

The accuracy of this process is assessed by the studies of recovery using spiking samples at four levels of strength: LOQ, 50%, 100%,150%, and 200% of the specification level, correlated with the strength 6.0ppm,30ppm, 60ppm, 90ppm and 120ppm, respectively. Table-6 denotes the data recovery, by the total three preparation responses at each and every spiked level, which are recorded. % RSD to total spiked levels is not more than that of the value 6.5%. The percentage recovery values so achieved vary

between 90% to 100%, which indicates that this proposed work accurately quantifies DMG foreign substance content in LID.

Table-6: Accuracy Results

Levels	Mean % Recovery (n=3)	%RSD
LOQ Level (6.05 ppm)	100.0	6.32
50% Level (30 ppm)	90.0	1.00
100% Level (60 ppm)	94.0	3.33
150% Level (90 ppm)	93.0	1.05
200% Level (120 ppm)	94.0	0.00

Range

Based on the findings, the operational range of the methods extends from LOQ to 150% level of specification. This range encompasses strength varies by LOQ 6.05 ppm to 2 times (120ppm) of the specified threshold, ensuring the method's suitability to QA across a broad spectrum of DMG foreign substance strength.

Robustness

A robustness study was carried out by varying the flow of the column as ±5% and, the temperature of the column by ±10°C, and Buffer Strength as ±10%, and the results obtained are within the acceptance criteria. The outcomes reported in Table-7.

Table-7: Robustness

Robustness condition	% RSD	Obtained result % RSD
Column flow 0.40mL/min	1.5	2.1
Column flow 0.38mL/min	3.7	2.0
Column flow 0.42mL/min	2.2	2.3
Column Temperature-44°C	1.9	1.3
Column Temperature-36°C	2.2	1.9
0.011M Ammonium formate	1.0	1.0
0.009 M Ammonium formate	1.7	1.7

Solution Stability

Keeping the solutions at a temperature of 25°C and the identified standard and spiked sample is highly stable in a time interval of 24 hrs.

Green Assessment for a Validated Method

Application of green belief for the newly developed process to appraise the greenness profile of this proposed work (compared to reported processes), as well-organized green metrics are adopted a qualitative tool, such as Analytical Greenness Metric (AGREE), as well as blue applicability grade index (BAGI) assessment tool.

AGREE Assessment Tool

The AGREE tool was refined in Poland by the Gdansk University of Technology. The AGRRE tool assesses 12 principles of greenness in analytical chemistry. The AGREE tool is illustrated as a circular pictogram divided into twelve segments, each and every segment portraying a distinct green principle. AGREE score is exposed at the center of the pictogram. If the toxic nature of the solvents is low, then the AGREE score is higher. In this AGRRE, which is less than 0.50, reveals that the approach is not adequate or environmentally friendly. The score ranges from 0.50 to 0.75. exposing the AGREE greenness tool, the newly evolving process score reported 0.61. The AGREE significance method is highly productive in terms of environmental legitimacy. The AGREE tool pictogram is shown in Fig.-6.

Blue Applicability Grade Index (BAGI) Assessment Tool

This tool is improved by Natalia Manousi, Wojciech Wojnowski, justynapolotka- wasylka and Victoria Samanidou. BAGI act as a correlative metric to green metrics, which primarily feature the repercussions of environmental analytical approach, besides the defense of solvent, generation of waste, consumption of

energy and so on. BAGI assessment is more integrated than other green assessment tools. This tool has two types of values: a score as well as an asteroid pictogram. Pictogram is divided into a total of ten segments, every segment representing a rendition criterion, as well as visually exhibits the method's compliance with these criteria utilising a hue scale: dark blue denoted as high acquiescence, blue denoted medium acquiescence, light blue represents low acquiescence, and white represents no acquiescence. Centerfield revealed as a score which varies between 25 and 100, with 25 indicating very poor interpretation, and 100 representing excellent interpretation. A score of at least 60 is recommended for the process to be considered practical. The BAGI green assessment tool was adopted for this DMG. The foreign substance content in LID drug substances optimized LC-MS method is 70.0, and the BAGI tool pictogram is shown in Fig.-6. The BAGI assessment highlighted the outstanding practical and significance of the technique.

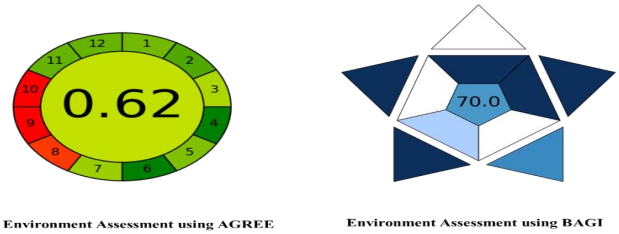

Fig.-6: AGREE and BAGI Tools

Similar quantifying studies on the Dimethylglyoxime Content in Lenalidomide Drug Substances are not available till now. Wong *et al.* in 2018[19] published a review article on LC-MS/MS Methods for Rapid Quantification of Oncology Drugs. Neugebauer *et al*. in 2018[20] worked on Quantification of the total nine Antimicrobials by LC-MS/MS to Therapeutic Drug Monitoring for critically ill patients.

CONCLUSION

LC-MS is successfully applied to routine analysis in many areas. The validated method addressed the challenge of quantifying DMG foreign substance in LID drug compounds, provides a reliable and validated process that meets rules and regulations, and significantly contributes to pharma QC. Chromatographic circumstances are fine-tuned to confirm the efficacious resolution of the DMG foreign substance, achieving precise retention times. These proposed separation strategies showed high specificity, substantiating clear resolution of DMG foreign substance by the drug compound, also with foreign substances. Moreover, this method is refined to provide breed values, minimizing matrix obstacle as well as ameliorating sensitivity. The greenness evaluation by AGREE and BAGI tools corroborates that this developed process maintains an optimal balance between the performance of the analyte and environmental responsibility. For validation of analytical procedures, considering the assessment of greenness to ensure the safety of analysts and the environment. The research presented in this paper comes under SDG-12 and SDG-13 because we described the content as green assessment and reasonable consumption.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest among the authors.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-17: Partnership For the Goals*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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